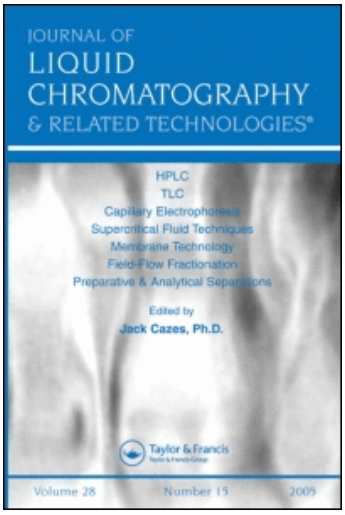

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THE INFLUENCE OF pH AND ACETONITRILE ON THE HIGH PERFORMANCE SIZE EXCLUSION PROFILE OF PROTEINS

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ABSTRACT

The concentration of the organic modifier, acetonitrile and pH were altered to measure their effects on the retention of various hydrophilic proteins and vitamin B_{12} using high performance size exclusion chromatography (HPSEC). The best separations were obtained with 0.1 or 0.2M sodium phosphate in the pH range of 6.0 to 7.0. In the absence of TFA, up to 25% acetonitrile did not dramatically influence the size exclusion profile of the protein components. Although 30 to 40% acetonitrile and 0.1% TFA for elution was not acceptable for the chromatography of hydrophilic proteins, hydrophobic apolipoproteins were nicely resolved under these conditions.

INTRODUCTION

Both pH and ionic strength influence the elution behavior of proteins in HPSEC for both silica based (1) and polymer based columns (2). Generally, retention times of biopolymers are reduced as the pH is lowered (2). The effect of pH has been attributed either to ion exchange (1) or hydrophobic effects (2) while that of peak broadening has been related to hydrophobic effects (1). The present study expands on these observations.

Some membrane bound and other hydrophobic biopolymers are best solubilized in organic solvents, in the absence of high concentrations (>.1M) of salt. This may limit the likelihood that they could be successfully chromatographed on most size exclusion columns since up to 0.1M salt is often recommended for HPSEC elution.

Size exclusion has been commonly used to purify membrane-bound and other hydrophobic proteins, as well as hydrophobic proteins in the presence of HPSEC detergents. HPSEC is also an effective technique for quantitating protein denaturation (3). Swergold and Rubin described the use of acetonitrile, water, and trifluoroacetic acid (TFA) such that when these eluents were used with polymer-based gel filtration columns, good resolution was obtained (4). An acetonitrile, water, TFA system was attempted in this study to elute both hydrophobic and hydrophilic proteins in a silica-based HPSEC column. In addition, pH and ionic strength were studied to determine the influence of these variables on the elution profiles of hydrophilic proteins in this column. Additional separations were carried out for hydrophobic apolipoproteins to test the usefulness of the technique for proteins which are insoluble in aqueous solvents.

Acetonitrile was chosen as an organic modifier because it is commonly encountered in HPLC. Trifluoroacetic acid (TFA) is also a common ion pairing agent. Although the influence of these modifiers in HPSEC is not clear, both have been used to reduce hydrophobic and ion exchange effects respectively. High concentrations of salt (0.01 to 0.1M) are also recommended for HPSEC to avoid ion exchange effects in the absence of TFA. The influence of these modifiers on the HPSEC of hydrophilic or hydrophobic proteins is not well understood.

MATERIALS AND METHODS

Both the Bio-Rad Quick Check Analyzer and Bio-Sil TSK 250 columns were obtained from Bio-Rad. Solvents and other reagents were of the highest purity available. HPLC grade solvents were used when available. The gel filtration protein standard consisting of a mixture of thyroglobulin, immunoglobulin, ovalbumin, myoglobin, and vitamin B_{12} was from Bio-Rad. Apolipoproteins were purchased from Sigma (St. Louis, MO). McIlvaine's citric acid phosphate bufer was used for pH analyses of the gel filtration standard (5). This involved mixing 0.1M citric acid with 0.2M disodium phosphate to obtain the desired pH. This buffer is effective in the pH range of 2.2 to 8.0 (5). The identification of peaks was carried out by eluting individual components. Water was deionized and distilled. Reagents were of the highest purity available.

RESULTS

The elution profile of a mixture of four proteins and vitamin B_{12} under chromatographic conditions which are recommended by the manufacturer is shown in Figure 1a. When an identical sample was chromatographed in this system using distilled water as the mobile phase (with the pH adjusted to 3.5 with HC1) the chromatotgram in Figure 1b resulted. Here, as well as when the elution buffer was switched to either 0.1% TFA (Figure 1c), 30% CH_3CN, 0.1% TFA (Figure 1d) or 0.1M KC1, 30% CH_3CN, 0.1% TFA (Figure 1e) all the proteins in the mixture eluted at or near the void volume, leaving the retention time of vitamin B_{12} and the porphyrin ring of myoglobin unchanged. The porphyrin ring of myoglobin eluted separately from the protein fraction only in the presence of CH_3CN (Figure 1).

Lowering the pH should protonate both the protein and the stationary phase in HPSEC. Proteins will also unfold under these conditions, complicating the

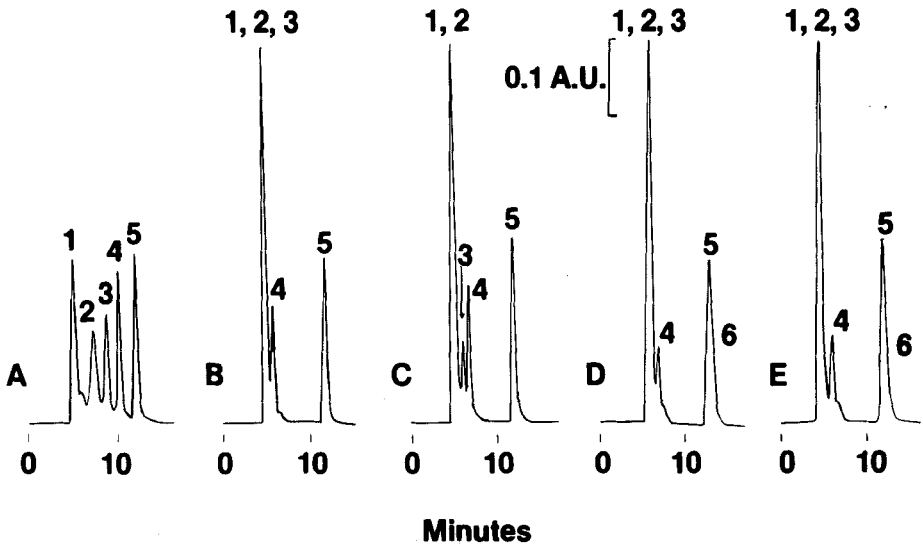

Figure 1. High performance size exclusion chromatography (HPSEC) of a protein standard (see Materials and Methods) using the Bio-Sil TSK-250 column under different eluants: a) 0.1M $NaPO_4$, pH 6.5, b) distilled water, pH 3.5 adjusted with HCl, c) 0.1% TFA, d) 0.1% TFA, 30% CH_3CN, and e) 0.1% TFA, 30% CH_3CN, 0.1M KCl. The flow rate was 1.0 ml.min and detection was using UV absorbance at 280 nm. Each chromatogram shows the retention times of 1) thyroglobulin, 2) gamma globulin, 3) ovalbumin, 4) myoglobin, 5) vitamin B_{12}, and 6) porphyrin. In the presence of acetonitrile, the porphyrin ring also apparently became isolated from myoglobin.

interpretation of the results in Figures 1a-d. If proteins were excluded by ionic repulsion, the addition of 0.1M KCl should have at least partially masked the effect. Figure 1e shows that 0.1M KCl had little or no influence on protein exclusion. Gradual adjustments in pH showed that each component of the protein mixture was influenced differently. While this can also be due to ion exclusion effects, it is less likely not only because of the failure of 0.1M KCl to dampen the exclusion effect, but also due to the poor correlation between isoelectric points for these proteins and shifts in retention with pH. The pI values for each of the proteins are listed in Table I.

In an effort to understand the role of pH in these elution profiles (Figure 1), a series of experiments were carried out in the pH range of 2.2 to 7.0 (Figure 2). McIlvaine's citric acid-phosphate buffer was used for these experiments (see Materials and Methods). It is clear from the chromatograms in Figure 2 that pH significantly influences the retention behavior of these proteins.

Table I
Isoelectric Points of Proteins*

Protein	pI
Thyroglobulin	4.5
Ovalbumin	4.7
Immunoglobulin	5.8-7.3
Myoglobin	6.5-7.5

*Values were from Righetti et al (6).

Table II

Elution Conditions (% CH_3CN)	Void Volume	IgG	Ovalb.	Myo.	B_{12}
	(Percent of protein eluting with native component)				
0	100	100	100	100	100
10	100	100	100	100	100
20	102	104	97	95	102
25	124	86	75	86	107
30	190	50	69	14	102

Values in this table are from areas of peaks shown in Figure 3. Elution conditions are defined in Figure 3. Initial areas for 0% CH_3CN were defined as 100% for each peak.

In the absence of TFA, acetonitrile alone does not dramatically influence the elution profile of these hydrophilic proteins (Figure 3). When 10% acetonitrile was included in the mobile phase, only the retention time of vitamin B_{12} was influenced (Figure 3b). With 20% acetonitrile, protein components may either aggregate or unfold and elute in the void volume (Figure 3c). Even in the presence of 20% CH_3CN, less than 5% of each protein eluted in the void peak. All of the IgG remained at the retention time of native IgG without aggregating in the presence of up to 20% CH_3CN (Table II). When 30% acetonitrile was included in the elution of buffer, most of the hydrophilic proteins eluted with the void peak (Figure 3, Table II).

While these hydrophilic proteins did not separate well on this column with acetonitrile and TFA (Figure 1), hydrophobic proteins may be less severely influenced by these conditions. An example is given in Figure 4 in which a chromatogram is shown of the separation of human apolipoproteins using 40%

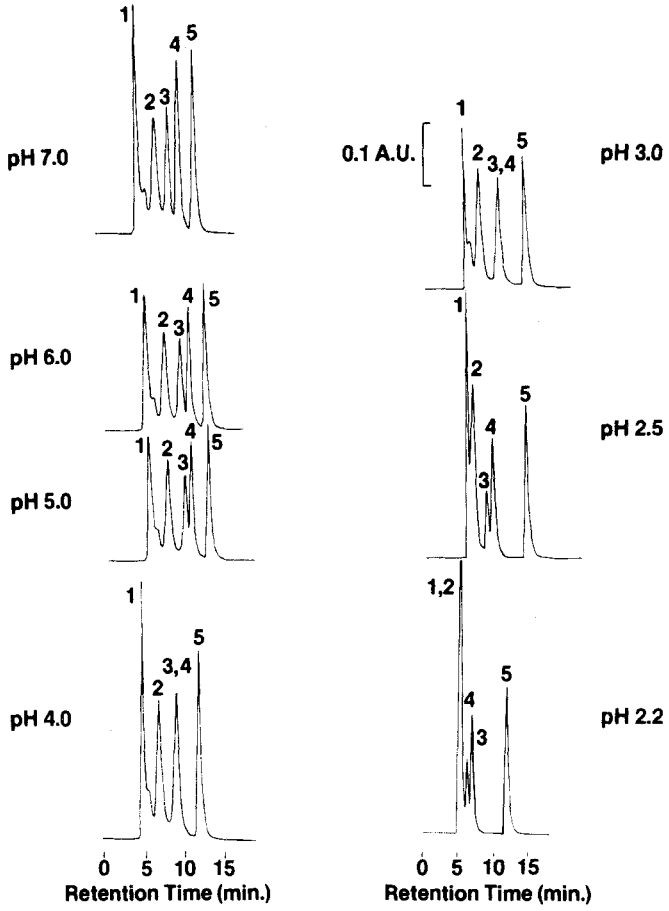

Figure 2. Conditions are as in Figure 1 except that the elution buffer was McIlvaine's citric acid-phosphate consisting of various mixtures of 0.1M citric acid and 0.2 M disodium phosphate, with pH adjusted as indicated. Peak identities are defined in Figure 1.

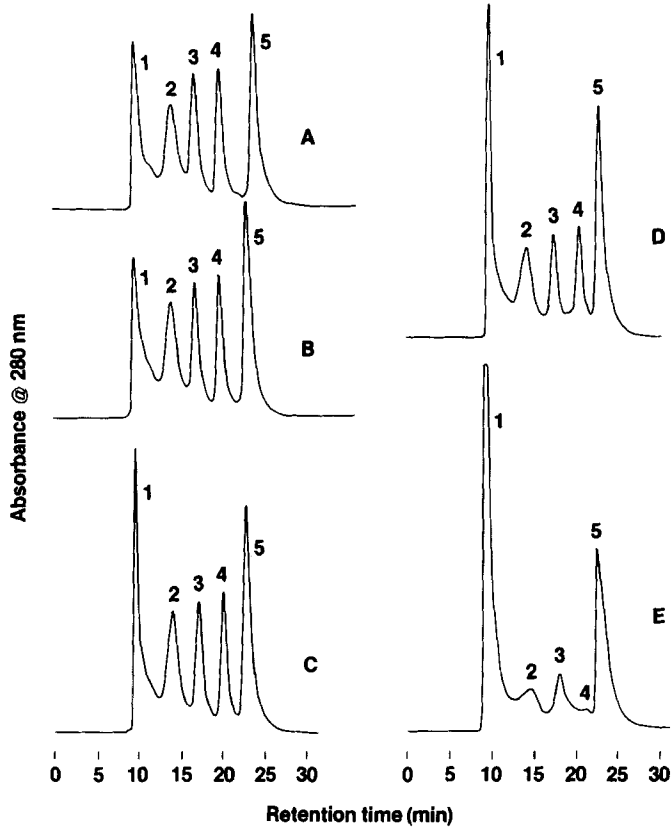

Figure 3. Size exclusion HPLC of hydrophilic proteins and vitamin B12 with different concentrations of acetonitrile. All chromatograms were run using 0.1M sodium phosphate (pH 6.5) and a) 0%, b)10%, c) 20%, d) 25%, and e) 30% acetonitrile. Other conditions were as described in Figure 1.

CH_3CN and 0.1% TFA in the mobile phase. This separation is nearly identical to those reported when SDS (7) or urea (8) were used in place of acetonitrile and TFA.

DISCUSSION

Initial studies were conducted using 0.1% TFA and 30% acetonitrile using a standard protein mixture (Figure 1d) to determine how these hydrophilic proteins behave under conditions in which hydrophobic polypeptides are likely to elute.

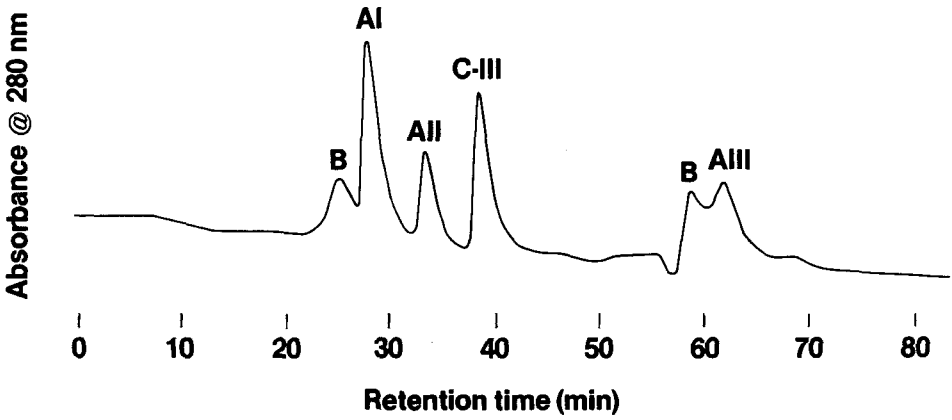

Figure 4. A mixture of human serum apolipoproteins was dissolved in 0.1% TFA and 40% CH_3CN. Twenty microliters of the mix were injected and run in an isocratic HPLC system equipped with two Bio-Sil TSK 250 columns connected in series (300 X 7.5 mm each). Flow rate was 0.4 ml/min. Peak identities were determined from injecting individual proteins under otherwise identical conditions. AI = apolipoprotein a_1, AII = apolipoprotein a_2, B = apolipoprotein b_1, C III = apolipoprotein c_3.

Further experiments were done to determine the cause(s) of the differences in the elution profiles between Figures 1a and d. Figure 1 demonstrates that independent of the presence or absence of salt or acetonitrile at low pH there was poor penetration of the hydrophilic protein mixture into the inner space of the column.

Elution pH had a strong influence on the retention time or apparent molecular weights of the hydrophilic proteins that were tested. Since retention times changed with pH at various rates for each protein, it is unlikely that these changes were due entirely to ionic effects. The isoelectric points also indicate that the proteins were not being excluded entirely by charge. It is more likely that extremes in pH caused alterations in tertiary structure, thus increasing the size and decreasing the retention of the proteins. Regardless of the mechanism, pH was a critical variable for the gel permeation of these hydrophilic proteins.

In the presence of acetonitrile, water, and TFA, apolipoproteins behaved as in SDS (7) or urea (8). This data suggests that the conformation of the apolipoproteins is similar in all three mobile phases. The acetonitrile water-TFA system may be preferable since it is volatile and UV transparent at 280 nm.

As the pH decreases, the proteins will become more positively charged. Thyroglobulin and immunoglobulin should be the last to be excluded from the HPSEC column if ion exclusion was the main force behind the early elution of these proteins. From Figure 2 it is clear that there are only slight differences in the exclusion of proteins with pH. The retention time of myoglobin appears to have diminished at the highest pH, as would be expected with only minimal adjustments in retention. Only at pH <<4.0, the proteins appeared to cluster at the void volume, as would be expected if they had unfolded with a larger apparent molecular weight.

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